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14. ABSTRACT Cyclin E is a positive regulator of the G1 to S phase transition of the cell cycle. In complex with CDK2 it is responsible for cells passing the restriction point, committing the cell to a round of the cell cycle progression. Previously this laboratory found that cyclin E is overexpressed and present in lower molecular weight (LMW) isoforms in breast cancer cells and tumor tissues compared to normal cells and tissues. To investigate the role of the LMW forms of cyclin E in tumorigenesis we are developing a model system of non-tumorigenic breast cells overexpressing the individual isoforms of cyclin E. Using this model system we aim to examine the role of the LMW forms in the process of transformation of normal cells in to neoplastic cells. We show that the LMW forms of cyclin E have higher kinase activity than the full length cyclin E. This activity is due to increased efficiency of binding of the LMW forms to the kinase subunit, CDK2, along with increased resistance to the inhibitors p21 and p27 compared to the full length cyclin E. Therefore, the LMW forms of cyclin E could play an important role in the pathogenesis of breast cancer.					
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Introduction:

The frequent appearance of the LMW forms of cyclin E and their correlation with poor prognosis in breast cancer patients indicate that the LMW forms may be oncogenic, playing specific roles in the development of malignancies. The purpose of this application is to determine the role of the LMW forms of cyclin E in the transformation of normal cells in to neoplastic cells. The outcome of this research will be significant in that it will delineate a novel mode of deregulation of cyclin E and possibly identify a new oncogene involved in breast cancer tumorigenesis therefore providing the rationale for novel drug design.

Body:

To determine the role of the low molecular weight (LMW) isoforms of cyclin E in tumorigenesis, we are generating a model system of immortalized, non tumorigenic mammary epithelial (76NE6) cells overexpressing each of the cyclin E isoforms. We will then characterize each of the clones overexpressing the individual isoforms and compare these cells to the untransfected and vector alone transfected cells. We have already shown that the LMW forms of cyclin E deregulate the cell cycle of non-tumorigenic mammary epithelial cells[1]. We will next assess how each of the clonal cell lines performs in assays that serve as surrogate indicators of tumorigenicity (for example, soft agar colony forming assays).

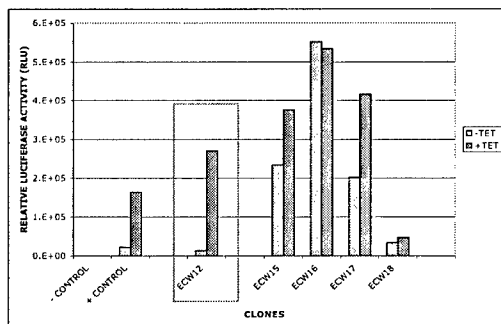


Figure 1: ECW12 is a clone of 76NE6 cells that contains the pTet-on vector allowing for inducible control of downstream genes. 76NE6 cells were transfected with the pTet-on vector that contains the tetracycline responsive transactivating domain. Cells were cotransfected with a puromycin vector and selected using 0.5µg/mL puromycin. Clones were then screened for the Tet-on vector by transiently transfecting the cells with pTRE-luciferase and assaying for reporter activity. Relative luciferase units (RLU) were determined with and without the addition of 1mg/mL of doxycycline (a tetracycline derivative). The negative control is untransfected 76NE6 cells. The positive control is commercially available

However, we have not been able to generate a clone stably expressing the T2 isoform. The non tumorigenic cells may not tolerate this form of cyclin E, therefore we have initiated an alternative strategy of cloning using a tetracycline inducible system. This will allow us to ultimately control the level of expression of our gene of interest. Generation of this model system requires the stable transfection of two plasmids, one containing the tetracycline transactivating domain, and a second containing a tetracycline responsive element and the gene of interest (cyclin E-EL, T1 or T2). We have generated a clone of 76NE6 cells stably expressing the first plasmid (figure 1). We are currently isolating and screening clones of 76NE6-ECW12 expressing the second, response element containing vector.

We aim to determine the role of the LMW forms of cyclin E in tumorigenesis. We have shown that the LMW forms of cyclin E exhibit higher kinase activity compared to the full length cyclin E (figure 2 and appendix)[2]. Therefore, it is important to decipher the biochemical differences between the cyclin E isoforms in order to understand the mechanisms

that provide the lower molecular weight isoforms their hyperactive phenotype and thus the potential for tumorigenicity. We have employed an insect cell (*Sf9*)

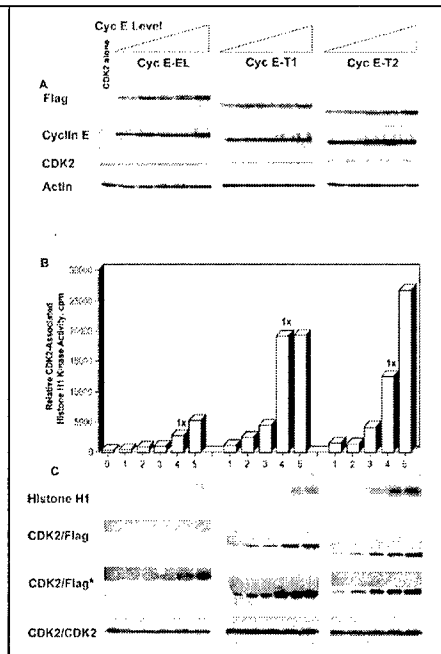


Figure 2: The LMW forms of cyclin E are hyperactive and bind more effectively to CDK2 than the full-length form of the protein. *Sf9* insect cells were co-infected with a set titer of Baculovirus containing CDK2 (0.7 MOI) and increasing MOI (0.125X, 0.25X, 0.5X, 1X and 2X) of Baculoviruses containing the three forms of cyclin E (EL, T1, or T2). Infection with CDK2 alone was used as a negative control. Sixty hours post-infection cell extracts were prepared for western blot analysis (A) and kinase assay (B) and IP assay (C). (A) 50 µg of cell extract was subjected to SDA-PAGE gels; the gels were then subjected to Western blot analysis with the indicated antibodies. (B) Relative CDK2-associated Histone H1 kinase activities were quantitatively investigated using 300 µg of protein extracts immunoprecipitated with polyclonal antibody CDK2 and then coupled to the Sepharose protein A beads. Each band in the autorad was quantified (cpm) and is presented here as raw counts. (C) 300 µg of protein extracts from each sample were also immuno-precipitated with polyclonal antibody CDK2 then coupled to the protein A beads before subjected to Western blot analysis using the monoclonal antibodies FLAG and CDK2. CDK2/Flag* indicates a longer exposure of the CDK2/FLAG. This figure is representative of 3 similar experiments.

model to examine the complexes formed by the cyclin E isoforms with CDK2 and the cyclin dependent kinase inhibitors, p21 and p27. With this model we can assess the binding of the cyclin E isoforms to these regulatory proteins and quantify the resulting kinase activity associated with the complexes. We have shown that the LMW forms of cyclin E appear to bind CDK2 more efficiently than the full-length cyclin E does (figure 3 and appendix). Furthermore, this analysis revealed that the IC₅₀ of p21 and p27 for full-length cyclin E-CDK2 against GST-Rb was 25 nM (Fig. 3D). In contrast, the concentrations of p21 and p27 required to inhibit the kinase activity of the LMW cyclin E-CDK2 by 50% were 3–5-fold higher. The trend was similar when HH1 was used as a substrate (EL required less of each CKI to inhibit its activity by 50% compared with T1 and T2),

but as would be expected, the differences between the isoforms was not as stratified due to the decreased specificity of the

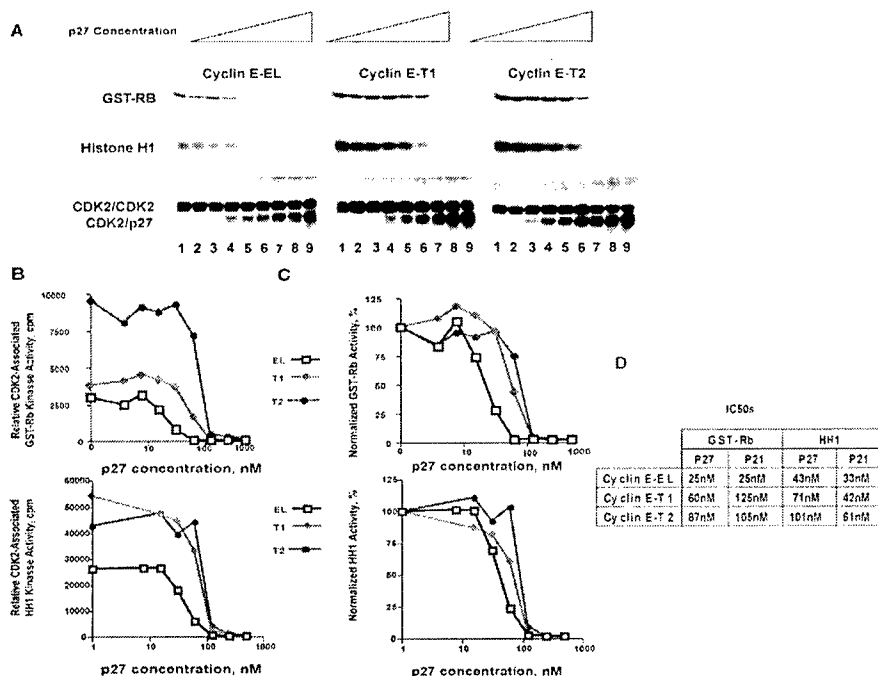


Figure 3: The LMW forms of cyclin E are resistant to inhibition by purified p27. Sixty hours after co-infection with 0.7 MOI of baculovirus containing CDK2 and the indicated cyclin E constructs (EL, T1, or T2), *Sf9* insect cell lysates were prepared to obtain the protein extracts. Equal amount (300 μ g) of protein extracts from each condition were first incubated with purified HA-p27 at different concentrations ranging from 0 up to 500 nM (*i.e.* 0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 nM), corresponding to lanes 1–9, respectively, for 30 min at 4 °C. The samples were then immunoprecipitated with polyclonal antibody to CDK2 and subjected to kinase assays using GST-Rb (A, top panel) or Histone H1 (A, middle panel) as substrates or to Western blot analysis with the indicated antibodies (A, bottom panel). The kinase assays were quantified and either presented as raw Cerenkov (*i.e.* cpm) values (B) or normalized to values of cyclin E-CDK2 kinase activity alone (without p27 inhibition) (C). IC50 values, corresponding to 50% inhibition of the CDK2 kinase activity, were extrapolated from C (D). The IC50 values for both p27 and p21 are shown. This figure is representative of triplicate experiments.

suggesting that the resistance of the LMW forms of cyclin E to CKIs is not due to higher basal activity of these forms as compared with the full-length. Western blot analysis suggests that there was no significant difference in the binding of the p27 to the full-length *versus* the LMW cyclin E-CDK2 complexes (*i.e.* Fig. 3A, lanes 4–6). These results suggest that the LMW cyclin E is much more resistant to inhibition by p27 than the full-length cyclin E. Furthermore, the differences in sensitivity to inhibition by CKIs between the full-length and LMW cyclin E is not merely due to the differences in the basal levels or activity of the LMW isoforms of cyclin E; nor is it due to differential binding properties of these proteins.

Key Research Accomplishments:

- Determined that the LMW forms of cyclin E bind CDK2 more efficiently than the full length cyclin E
- Ascertained the LMW forms of cyclin E are resistant to inhibition by p21 or p27
- Established a cell line of non-malignant mammary epithelial cells stably expressing the tetracycline-transactivating domain so that expression of a gene of interest (such as the LMW isoforms) can be induced and controlled.

Reportable Outcomes:

The following manuscript has been published: Please see appendix for copy.

- Wingate H, Zhang N, McGarhen MJ, Bedrosian I, Harper JW, Keyomarsi K.; The tumor-specific hyperactive forms of cyclin E are resistant to inhibition by p21 and p27; J Biol Chem. 2005 Apr 15;280

Posters were presented at the following meetings:

- Comparative Mouse Genomics Centers Consortium Symposium 06/04
- AACR- Cell Cycle Meeting 12/04
- AACR Annual Meeting 03/04
- Department of Defense Breast Cancer Research Program Era of Hope 06/05

Conclusions:

The LMW isoforms of cyclin E are specific to tumor cells. Furthermore, overexpression of the LMW isoforms of cyclin E is indicative of breast cancer progression. Therefore it is crucial to both the fields of cell cycle biology and breast cancer research to understand the mechanism of action of the LMW forms of cyclin E. We have shown that the LMW forms of cyclin E have higher kinase activity than the full length cyclin E. This can be attributed to higher efficiency of binding of the LMW forms of cyclin E to CDK2. In addition, the LMW forms appear to be resistant to inhibition by the CDK inhibitors p21 and p27. With higher activity and resistance to the predominant form of negative regulation of the activity it could be perceived that the LMW forms of cyclin E are the machinery necessary for uncontrolled growth regulation of a cell and the possibility for a neoplastic phenotype.

References:

1. Wingate, H., et al., *The low molecular weight (LMW) isoforms of cyclin E deregulate the cell cycle of mammary epithelial cells*. Cell Cycle, 2003. **2**(5): p. 461-6.
2. Wingate, H., et al., *The tumor-specific hyperactive forms of cyclin E are resistant to inhibition by p21 and p27*. J Biol Chem, 2005. **280**(15): p. 15148-57.

The Tumor-specific Hyperactive Forms of Cyclin E Are Resistant to Inhibition by p21 and p27*

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The low molecular weight (LMW) isoforms of cyclin E are unique to cancer cells. In breast cancer, such alteration of cyclin E is a very strong predictor of poor patient outcome. Here we show that alteration in binding properties of these LMW isoforms to CDK2 and the CDK inhibitors (CKIs), p21 and p27, results in their functional hyperactivity. The LMW forms of cyclin E are severalfold more effective at binding to CDK2. Additionally, compared with the full-length cyclin E-CDK2 complexes, the LMW cyclin E-CDK2 complexes are significantly more resistant to inhibition by p21 and p27, despite equal binding of the CKIs to the LMW complexes. When both the full-length and the LMW cyclin E are co-expressed, p27 preferentially binds to the LMW forms yet is unable to inhibit the CDK2 activity. Thus, the LMW forms of cyclin E may contribute to tumorigenesis through their resistance to the inhibitory activities of p21 and p27 while sequestering these CKIs from the full-length cyclin E.

Normal cell division is precisely regulated by checkpoints at distinct junctures in the cell cycle (1). The G₁/S checkpoint is invariably deregulated in human tumors (2) and is therefore relevant to the process of carcinogenesis. Cells in G₁ are normally responsive to extracellular mitogenic stimulation driving them into quiescence or into another round of proliferation (3). In cancer cells, this dependence on exogenous signals is uncoupled through a potential myriad of mutations, preventing the cells from exiting the cell cycle (2). Central to the passage of cells through G₁ into S phase is the Rb pathway (4). This pathway is positively regulated through sequential phosphorylation of Rb by cyclin-dependent kinases (CDKs)¹ and their associated cyclins. The cyclin D-CDK4 and cyclin D-CDK6 complexes are important in early G₁ for phosphorylation of Rb

(5–9). The resultant uncoupling of E2F from Rb allows E2F to initiate the transcription of cyclin E and additionally the transcription of S phase genes (10–12). Cyclin E in association with CDK2 continues the phosphorylation of Rb, with its levels peaking around the restriction point late in G₁ (4, 13, 14). This progression through the cell cycle is also negatively regulated by inhibitors of the CDKs (CKIs) such as those of the INK4 family (p16, p15, p18, and p19) that inhibit CDK4 and CDK6 and the Cip/Kip family (p21 and p27) that inhibit CDK2 (reviewed in Ref. 15). Mutations in the cyclins, CDKs, and CDK inhibitors that regulate the RB pathway as well as mutations of Rb itself have been described in most human cancers (2).

The tight regulation of cyclin E both at the transcriptional level and by ubiquitin-mediated proteolysis indicates that it has a major role for the control of G₁/S transition. The recent identification of key substrates for cyclin E-CDK2 complex has increased our appreciation on how cyclin E overexpression seen in many human cancers can lead to genomic instability, a feature common to most solid malignancies. We and others have previously reported that the cyclin E gene is amplified in some breast cancer cell lines (16, 17). This amplification can result in as much as 64-fold overexpression of cyclin E mRNA and the protein product, which is constitutively expressed across all phases of the cell cycle (18, 19). In addition to overexpression of the full-length cyclin E 50-kDa protein (found in both normal and tumor cells), we have discovered that some breast cancer cell lines and human breast cancers also express up to five lower molecular weight isoforms of cyclin E (ranging in size from 34 to 49 kDa) (16, 20–22). We have recently identified the mechanism that is responsible for generating the low molecular weight forms of cyclin E as elastase-mediated cleavage of the N terminus of the full-length cyclin E protein (23). We designate the full-length, 50-kDa form of cyclin E as EL. EL2 and EL3 are formed by elastase cleavage between amino acids 40 and 45 of the cyclin E protein followed by further post-translational modification, probably phosphorylation of EL3 to yield EL2. Together, EL2 and EL3 are collectively termed T1. Likewise, EL5 and EL6 are also formed by proteolytic cleavage by elastase at A69 with EL5 containing further post-translational modification, and these two forms are collectively termed T2. In contrast, EL4 is generated from an alternate start site at M46. Of note, EL2,3 (*i.e.* T1) and EL5,6 (*i.e.* T2) but not EL4 are expressed only in breast cancer cells with the highest levels of expression found in estrogen receptor-negative tumors.

The low molecular weight forms of cyclin E are hyperactive compared with the full-length form both biochemically and biologically. Both T1 and T2 can more efficiently phosphorylate substrates such as histone HH1 or GST-Rb compared with the

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¹ The abbreviations used are: CDK, cyclin-dependent kinase; GST, glutathione S-transferase; LMW, low molecular weight; MOI, multiplicity of infection; IP, immunoprecipitation; HA, hemagglutinin; CKI, CDK inhibitor; HH1, histone H1.

EL, full-length form, of cyclin E. This hyperactivity associated with the low molecular weight (LMW) forms of cyclin E facilitates the deregulation of the G₁ to S transit compared with the full-length form of the protein (23), thus providing a growth advantage to tumor cells. Cytogenetic analysis of MCF-7 cells overexpressing the LMW forms (T1 and T2) of cyclin E shows an increase in polyploidy and tetraploid cells in which the chromosomes contain significantly more structural aberrations (including breaks, fragments, and fusions) along with increased endoreduplication compared with MCF-7 cells overexpressing the full-length (EL) form of cyclin E. Additionally, the cells overexpressing the LMW forms of cyclin E became resistant to anti-estrogen (ICI 182,780) treatment, whereas those expressing the full-length cyclin E remain sensitive (24).

The LMW forms of cyclin E are also a significant predictor of poor outcome in breast cancer (25). Cyclin E levels in tumor tissue from 395 patients across all stages of disease were measured using Western blot assay and scored for expression of full-length and LMW isoforms. These levels were then correlated with other established prognostic factors and with disease-specific and overall survival. Cyclin E levels proved to be the most powerful independent predictors for survival in stage I to III breast cancer (25).

The identification of LMW forms of cyclin E generated specifically in tumors due to elastase mediated amino-terminal proteolytic processing poses new questions about the mechanism that underlies their biochemical properties compared with the full-length cyclin E. In this report, we delineate the biochemical differences in activity of the full-length cyclin E compared with its LMW forms with respect to their binding partners, CDK2, p21, and p27, using a baculovirus insect expression system and in tumor tissues from breast cancer patients. Our results show that the LMW cyclin E-CDK2 complexes are significantly more resistant to inhibition by p21 and p27 both *in vitro* using purified CKIs and *in vivo* when the CKIs were co-infected with cyclin E and CDK2. Furthermore, we show that under conditions where both the full-length and the LMW forms of cyclin E are present, p21 preferentially binds to the LMW forms of cyclin E and is therefore incapable of inhibiting the full-length cyclin E. Last, we show that breast cancer cell lines and tumors from breast cancer patients overexpressing LMW cyclin E, despite high expression and adequate binding of p21 and p27, do not get inhibited by the CKIs. These results suggest that one of the mechanisms by which the LMW forms of cyclin E mediate their oncogenic potential is through resistance to inhibition by p21 and p27.

EXPERIMENTAL PROCEDURES

Culture Conditions for Sf9 Cells.—Insect Sf9 cells were cultured in Grace's TNM-FH medium supplemented with 10% fetal calf serum in a 27 °C incubator in spinner cultures. For infections, 2×10^7 cells were plated in each 150-mm tissue culture plate and allowed to attach, and medium was changed before infection.

Plasmids, Virus Stocks, and Infections.—Plasmids pVLCCK2, pV-Lhp27, pVLP21, pVLCycE-EL, pVLCycE-T1, and pVLCycE-T2 were constructed by subcloning the full-length cDNA fragments for CDK2, human p27, p21, the EL forms of cyclin E, and its truncated forms (i.e. T1 and T2), separately, into the multiple cloning sites downstream of the polyhedrin promoter of BaculoGold virus transfer vector pVL1392 (PharMingen, San Diego, CA) to generate the expression vectors. Recombinant viruses were produced by co-transfection of these purified plasmids, separately, with linearized BaculoGold virus DNA (PharMingen) into Sf9 insect cells. For the resultant recombinant viruses (CDK2, EL, T1, T2, and p27), titers were determined by end point dilution assays. Viruses were amplified by infecting the Sf9 insect cells at a low multiplicity of infection (MOI) (0.7) to obtain a high percentage (99%) of the recombinant virus products in the large stock. To obtain protein extracts, cells were scraped off of plates 60 h postinfection, and the supernatants of cell medium containing the amplified viruses were harvested after centrifugation (1100 rpm, 10 min, 4 °C) and then stored

in 4 °C. Cell pellets were resuspended in 250 μ l of $1 \times$ ppi (250 μ g/ml leupeptin, 25 μ g/ml aprotinin, 10 μ g/ml pepstatin, 1 mM benzamide, 10 μ g/ml soybean trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride made in Me₂SO, 50 mM NaF, 0.5 mM sodium orthovanadate) and lysed by sonication. High speed supernatants of the lysates were then subjected to Western blotting, immunoprecipitation (IP), histone H1, and GST-RB kinase analysis, as described below.

For *in vitro* studies, co-infection of the recombinant BaculoGold viruses (CDK2 + cycE-EL, CDK2 + cycE-T1, and CDK2 + cycE-T2) were carried out to obtain the purified protein complexes of CDK2-EL, CDK2-T1, and CDK2-T2. The two desired viruses were mixed together at the same titer and used to co-infect the Sf9 cells at an MOI of 0.7 each. For controls, Sf9 were infected with the CDK2 virus alone. The viral load of the cells was kept constant by the addition of an empty, control vector. For the dose escalation studies, cells were infected with 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 MOI.

For *in vivo* studies, Sf9 cells were infected with three different viruses simultaneously (i.e. CDK2, cyclin E-T1 (or cyclin E-T2), and p27 (or p21)) in order to generate the following protein complexes: CDK2-CycEL-p27 (or p21), CDK2-T1-p27 (or p21), or CDK2-T2-p27 (or p21). The recombinant viruses of CDK2 and the three forms of cyclin E, respectively, were mixed at the same MOI, whereas p27 (or p21) was added in a series of different titers from 0, 0.25, 0.5, 1, 2, and up to 4 MOI compared with the titer of CDK2.

For sequestration study, Sf9 cells were infected with four different viruses simultaneously (i.e. CDK2, cyclin E-EL, cyclin E-T1 (or cyclin E-T2), and p27). The same titer of CDK2, cyclin E-EL, and cyclin E-T1 (or cyclin E-T2) was mixed and added onto Sf9 cells. Two different titers of recombinant viruses p27 (i.e. 1 and 2 MOI of CDK2) were added to investigate the competition. Co-infection of recombinant viruses of CDK2 and cyclin E full-length or its LMW forms (i.e. in the absence of p27 virus) as well as tri-infection of recombinant viruses of CDK2, cyclin E full-length (or its LMW forms), and p27 were also carried out on the same batch of Sf9 cells under the same incubation conditions to serve as controls. For all studies, infections were set up at least three times, and the lysates were consequently subjected to the appropriate experiments described below. Because the results were consistently similar, the figures in this paper are representative of the replicate experiments performed.

Western Blot, Immunoprecipitation, and H1 Kinase Analysis.—Western blot analysis was performed as previously described (23). Briefly, 50 μ g of protein extracts from each infection condition was electrophoresed on a 10% (FLAG or actin) or 13% (CDK2, p27, p21, and actin) of SDS-polyacrylamide gels, transferred onto Immobilon polyvinylidene difluoride membrane, blocked overnight at 4 °C with Blotto (5% nonfat dry milk in 20 mM Tris, 137 mM NaCl, 0.25% Tween 20, pH 7.6), washed with TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween 20, pH 7.6), and then incubated with anti-FLAG monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 0.75 μ g/ml, monoclonal antibodies to CDK2, p27 (PharMingen, San Diego) at 1 μ g/ml, monoclonal antibody to p21 (Oncogene Research Products/Calbiochem) at 1 μ g/ml, and monoclonal antibody to actin (Roche Applied Science) at 0.2 μ g/ml, all diluted in Blotto. Blots were developed with the Renaissance chemiluminescence system as instructed by the manufacturer (PerkinElmer Life Sciences).

Immunoprecipitation (IP) assays were performed as previously described (23). Briefly, 300 μ g of protein extracts from each condition was immunoprecipitated with anti-CDK2 polyclonal antibody, coupled to Sepharose-protein A beads, washed four times (6000 rpm, 3 min, 4 °C) with $1 \times$ lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, $1 \times$ ppi), followed by four washes with $1 \times$ H1 wash buffer (10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 25 mM Tris-HCl, pH 7.5, 125 mM NaCl). The immunoprecipitates were then electrophoresed on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with anti-CDK2 or anti-FLAG monoclonal antibody at 1 μ g/ml in Blotto and developed as described above.

For *in vitro* binding assays, purified HA-p27 or HA-p21 (26) was added, in a series of nine different concentrations from 0 up to 500 μ M, to 300 μ g of total cell lysate and incubated at 4 °C for 30 min followed by IP with anti-CDK2 antibody and coupling to protein A-Sepharose beads. The HA-p21 and HA-p27 complexes were then subjected to Western blot analysis with either anti-CDK2, anti-p27, or anti-p21 monoclonal antibodies, diluted at 1 μ g/ml in Blotto.

For the sequestration experiments, 300 μ g of protein extracts were first immunoprecipitated with polyclonal anti-p27 antibody (sc-528; Santa Cruz Biotechnology) and then incubated with Sepharose-protein A beads and subjected to Western blot analysis with anti-FLAG or anti-p27 monoclonal antibodies, diluted at 1 μ g/ml in Blotto.

Kinase assays were performed as previously described (23). Briefly, 300 μ g of protein extract from each condition was used, and following IP with CDK2, p21, or p27, the immunoprecipitates were incubated with 30 μ l of kinase assay reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 70 mM NaCl, 0.1 mg/ml bovine serum albumin) that contained 60 μ M cold ATP, 5 μ Ci of [³²P]ATP, and 5 μ g of histone H1 (Roche Applied Science) or 1.5 μ g of GST-Rb (Santa Cruz Biotechnology) at 37 °C for 30 min. The reaction products were then analyzed on 13% SDS-polyacrylamide gels (for histone H1) or 10% SDS-polyacrylamide gels (for GST-Rb), stained, destained, dried, and finally exposed to x-ray films. For quantitation purposes, the protein bands corresponding to histone H1 or GST-Rb were then excised and measured for their radioactivity by Cerenkov counting.

Cell Culture and Transient Transfection of Human Breast Cancer Cells—T47-D human breast cancer cells were cultured as described elsewhere (27). Empty vector pcDNA 4.0 (Invitrogen), cyclin EL1-FLAG, and cyclin E-FLAG constructs Trunk1 (T1) and Trunk2 (T2), described elsewhere (24), were transfected into T47-D cells by using Fugene (Invitrogen). Twenty-four hours following transfection, the cells were harvested and subjected to Western blot, immunoprecipitation, and kinase analysis as described above.

Breast Tumor Tissue Homogenate Preparation—Approximately 0.1–0.5 mg of the specimen was added to 1 volume of sonication buffer containing protease and phosphatase inhibitors, as described (28). The specimen was then homogenized in a tissue press (Micro-Mincer; Bio-Spec Products, Bartlesville, OK), followed by sonication at 4 °C using a cup-horn adapter. Homogenates were centrifuged at 100,000 $\times g$ for 45 min at 4 °C. The supernatants were assayed for protein content, aliquoted, and stored at –70 °C. 25 μ g of lysate were subjected to Western blot analysis using the monoclonal antibodies to cyclin E (HE12) at a concentration of 0.1 μ g/ml (29), p27 (Pharmingen, San Diego, CA), proliferating cell nuclear antigen (Santa Cruz Biotechnology), and actin (Roche Applied Science) at final concentrations of 1 μ g/ml in Blotto. Immunoprecipitations were also performed using 150 μ g of lysate and polyclonal p27 antibody (Santa Cruz Biotechnology), followed by Western blots using the cyclin E and p27 antibodies. Histone H1 kinase assays were performed using 300 μ g of lysate and polyclonal cyclin E antibody. The reaction products were then analyzed on 13% SDS-polyacrylamide gels (for histone H1) stained, destained, dried, and finally exposed to x-ray films.

RESULTS

The LMW Forms of Cyclin E Are Hyperactive and Have Increased Affinity for CDK2—Using a eukaryotic expression system, we examined the biochemical attributes of LMW cyclin E that could contribute to their enhanced function. For this purpose, we overexpressed full-length cyclin E, its LMW forms, and CDK2 in insect cells using the baculovirus expression system (Fig. 1). Three different cyclin E baculoviruses expressing each of the three FLAG-tagged cyclin E constructs termed EL, T1, or T2 were generated in insect cells. The EL construct codes for the full-length 50-kDa form of cyclin E, the T1 construct codes for the 45- and 44-kDa forms, (termed EL2 and EL3), and the T2 construct codes for the 35- and 33-kDa forms (termed EL5 and EL6). Hence, the forms generated by the T1 and T2 constructs correspond to the endogenous LMW forms that we termed EL2,3 and EL5,6, respectively, in breast cancer cell lines (23). Insect cells were co-infected with a constant titer (0.7 MOI) of recombinant baculovirus containing CDK2 and increasing MOI (0.125–2) of either FLAG-tagged cyclin E full-length (cycE EL), cyclin E-T1 (cycE T1), or cyclin E-T2 (cycE T2) cDNAs (Fig. 1). Sixty hours post-infection, cells were harvested and subjected to Western blot (Fig. 1A), histone H1 kinase (Fig. 1B), and immunoprecipitation (Fig. 1C) analyses. Western blot analysis with FLAG and cyclin E confirmed an increasing level of expression of cyclin E-FLAG (EL, T1, or T2), corresponding to increasing MOI (*i.e.* 0.125–2). Insect cells infected with CDK2 alone (*i.e.* no co-infection with cyclin E) only express CDK2, as depicted on Western blot analysis (Fig. 1A, CDK2 alone lane). Expression of CDK2 shows a slight increase in the CDK2 levels in cyclin E T1-infected cells. However, immunoprecipitation with CDK2, followed by CDK2 Western blot analysis, clearly shows that equal amounts of

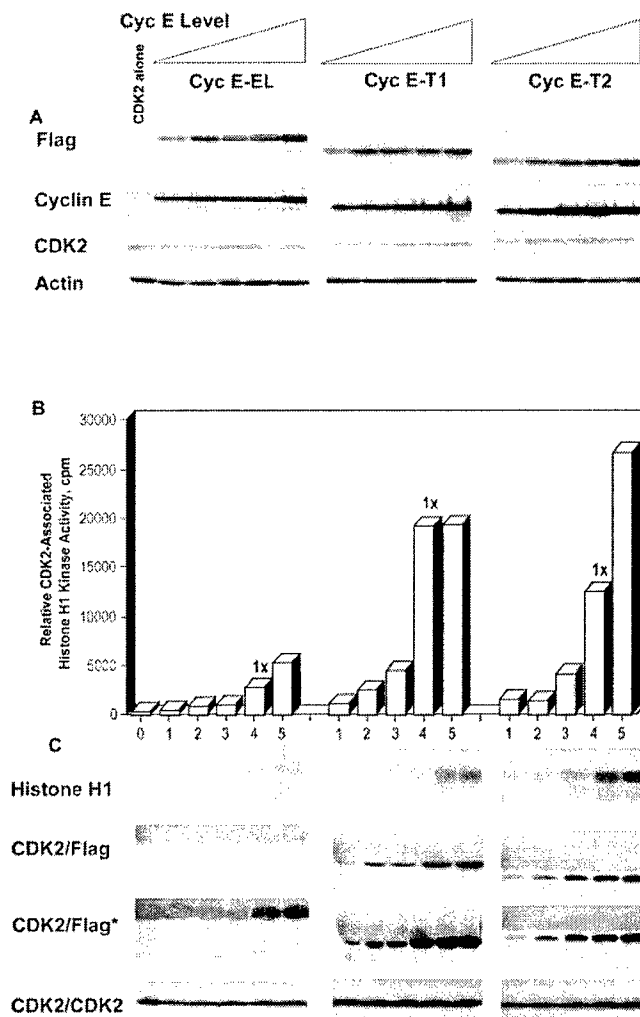


FIG. 1. The LMW forms of cyclin E are hyperactive and bind more effectively to CDK2 than the full-length form of the protein. Sf9 insect cells were co-infected with a set titer of baculovirus containing CDK2 (0.7 MOI) and increasing MOI (0.125, 0.25, 0.5, 1 and 2 corresponding to lanes 1–5) of baculoviruses containing the three forms of cyclin E (EL, T1, or T2). Infection with CDK2 alone was used as a negative control. Sixty hours post-infection, cell extracts were prepared for Western blot analysis (A) and kinase assay (B) and IP assay (C). A, 50 μ g of cell extract was subjected to SDS-polyacrylamide gels; the gels were then subjected to Western blot analysis with the indicated antibodies. B, relative CDK2-associated histone H1 kinase activities were quantitatively investigated using 300 μ g of protein extracts immunoprecipitated with polyclonal antibody CDK2 and then coupled to the Sepharose-protein A beads. Each band in the autorad was quantitated (cpm) and is presented here as raw counts. C, 300 μ g of protein extracts from each sample were also immunoprecipitated with polyclonal antibody CDK2 and then coupled to the protein A beads before being subjected to Western blot analysis using the monoclonal antibodies FLAG and CDK2. CDK2/FLAG* indicates a longer exposure of the CDK2/FLAG. This figure is representative of three similar experiments.

CDK2 were immunoprecipitated from each sample independent of the level of expression of cyclin E (Fig. 1C). Kinase assays using histone H1 as substrate showed that at each of the viral titers examined, the truncated forms of cyclin E have greater kinase activity than the EL form (Fig. 1C). After qualitative evaluation of the kinase blots, raw Cerenkov counts were obtained by scintillation counting to quantitatively confirm that the LMW forms of cyclin E have greater kinase activity than the full-length form (Fig. 1B). For example, in the 1 MOI infected cells (Fig. 1B, compare lane 4 in the three panels) the CDK2 activities associated with cycE-T1 and cycE-T2 were 7-

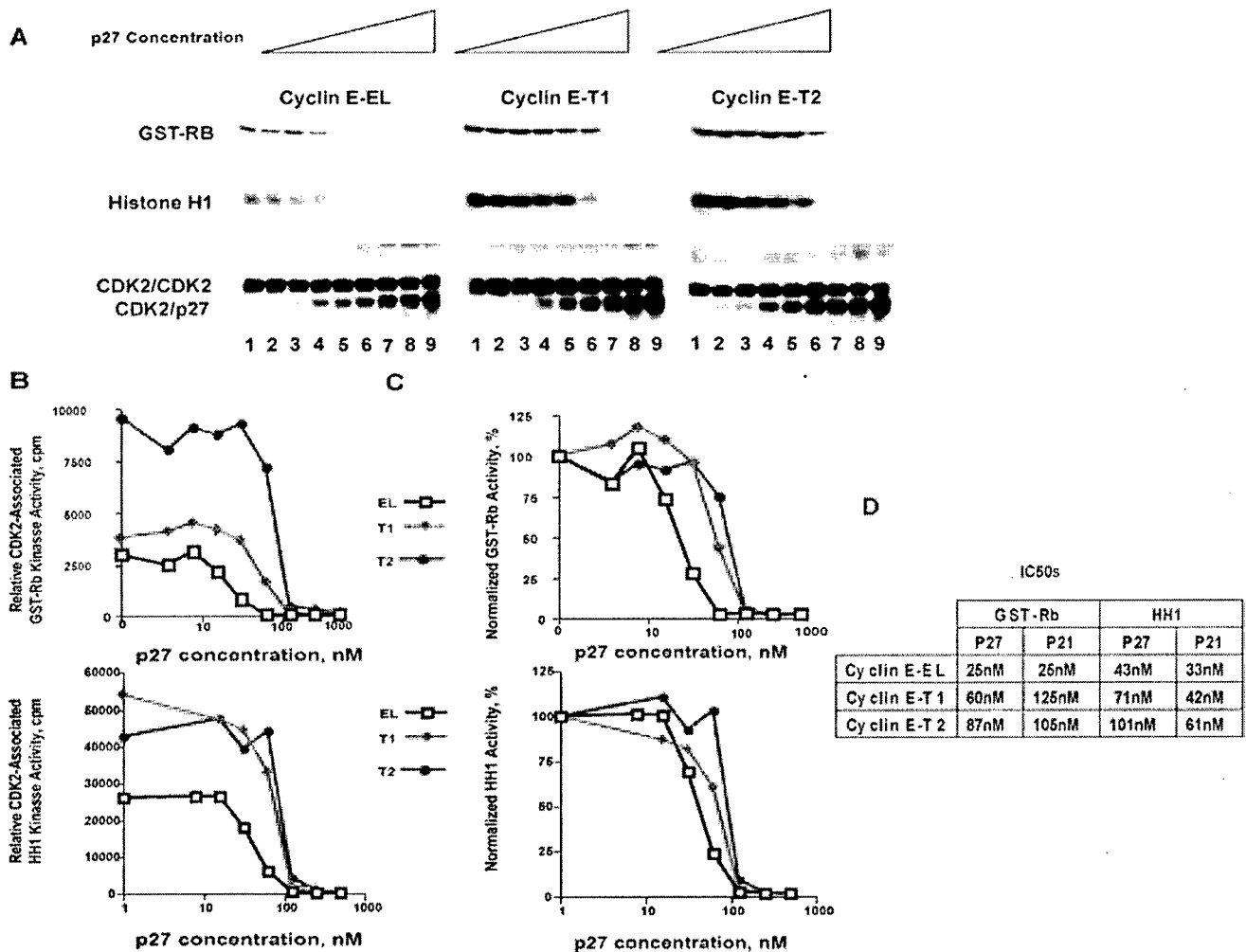


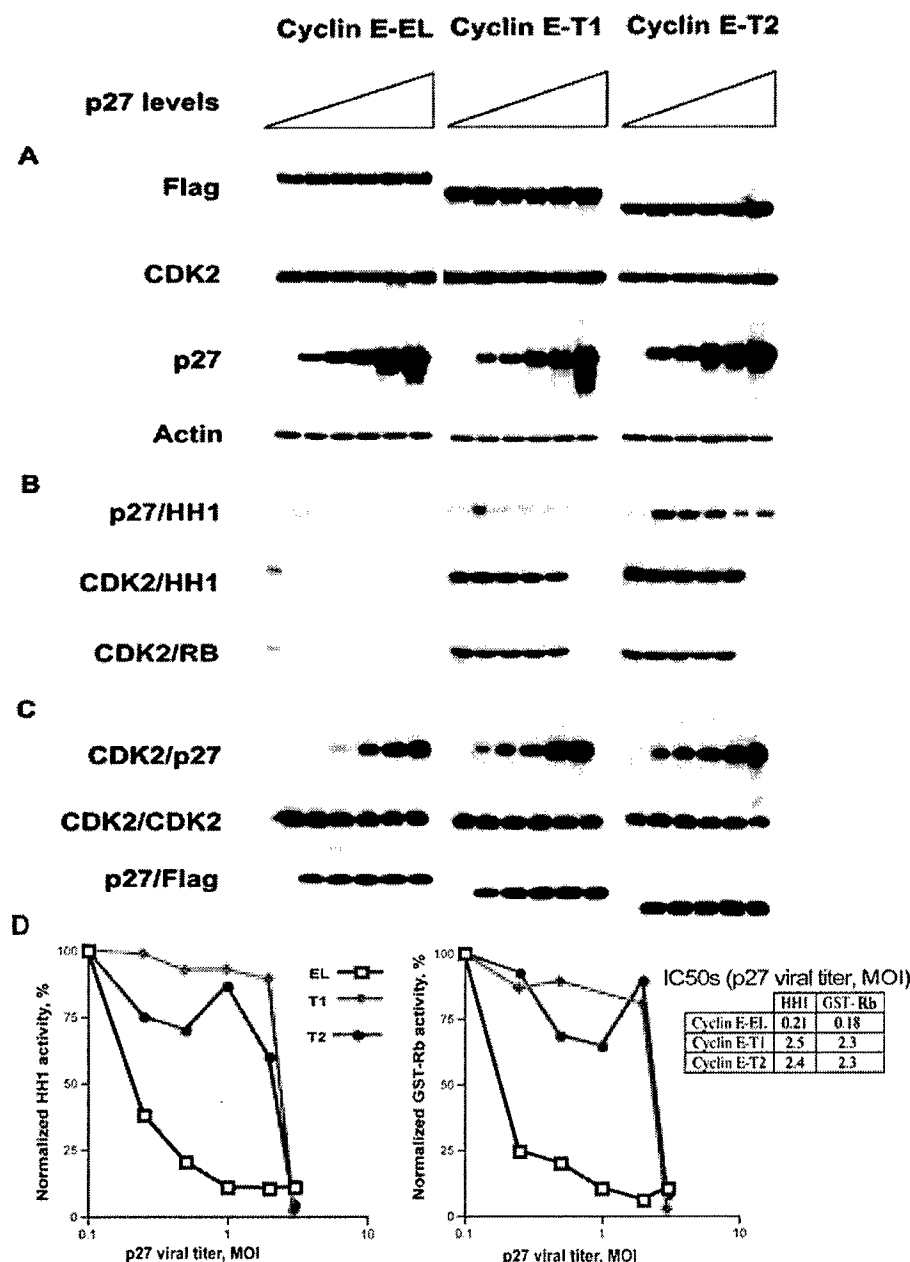
FIG. 2. The LMW forms of cyclin E are resistant to inhibition by purified p27. Sixty hours after co-infection with 0.7 MOI of baculoviruses containing CDK2 and the indicated cyclin E constructs (EL, T1, or T2), *Sf9* insect cell lysates were prepared to obtain the protein extracts. Equal amount (300 μ g) of protein extracts from each condition were first incubated with purified HA-p27 at different concentrations ranging from 0 up to 500 nM (i.e. 0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 nM), corresponding to lanes 1–9, respectively, for 30 min at 4 °C. The samples were then immunoprecipitated with polyclonal antibody to CDK2 and subjected to kinase assays using GST-Rb (A, top panel) or histone H1 (A, middle panel) as substrates or to Western blot analysis with the indicated antibodies (A, bottom panel). The kinase assays were quantitated and either presented as raw Cerenkov (i.e. cpm) values (B) or normalized to values of cyclin E-CDK2 kinase activity alone (without p27 inhibition) (C). IC₅₀ values, corresponding to 50% inhibition of the CDK2 kinase activity, were extrapolated from C (D). The IC₅₀ values for both p27 and p21 are shown. This figure is representative of triplicate experiments.

and 10-fold higher, respectively, compared with the activity associated with 1 MOI cycE-EL. Overall, in the range of viral titers examined, cycE-T1 and cycE-T2 phosphorylated histone H1 between 2- and 12-fold more effectively than the full-length cyclin E. CDK2 immunoprecipitation analysis revealed that the hyperactivity of the LMW forms of cyclin E was due to their enhanced ability to bind to CDK2 compared with the EL cyclin E. This binding of LMW cyclin E to CDK2 was seen across all titer levels (Fig. 1C). The CDK2 immunoprecipitates that were probed with FLAG are shown after two exposures (the lane with an asterisk denotes a longer exposure time). This was necessary to differentiate the weak binding of the full-length cyclin E from the interference of the IgG band. These experiments suggest that one reason that the LMW forms are hyperactive in their function is through more effective binding to CDK2 compared with the full-length protein.

The LMW Forms of Cyclin E Are Resistant to Inhibition by p21 and p27—Next, we characterized the binding of the LMW cyclin E isoforms to the CKIs p21 and p27 (Fig. 2). For these experiments insect cells were co-infected with recombinant

baculovirus containing equal amounts of CDK2 and either cyclin E full-length (CycE-EL), T1 (CycE-T1), or T2 (CycE-T2) cDNAs, all at 0.7 MOI. The co-infected insect cells were then homogenized and incubated with increasing concentrations (0–500 nM) of purified HA-p27 (Fig. 2) or HA-p21 (data not shown) for 30 min. The CDK2 complexes were then affinity-purified and subjected to Western blot analyses and kinase assays using either histone H1 or GST-Rb as substrates. All analyses were performed with both HA-p21 and HA-p27. Since very similar results were obtained with both CKIs, we are only showing the detailed analysis of HA-p27. These analyses show that the kinase activity associated with the full-length cyclin E-CDK2 complexes could be readily inhibited by purified HA-p27 using either histone H1 or GST-Rb as substrates (Fig. 2, A–C). Since equal amounts of the LMW isoforms have increased kinase activity compared with equal amount of full-length cyclin E, the kinase activity at each dose of p21 or p27 against the uninhibited cyclin E-CDK2 complex activity was normalized to account for their intrinsic differences in activity (Fig. 2C). This analysis revealed that the IC₅₀ of p21 and p27 for full-length

FIG. 3. The LMW forms of cyclin E bind to, but are resistant to inhibition by, p27 or p21 *in vivo*. *Sf9* insect cells were tri-infected concurrently with recombinant Baculoviruses containing p27 (A–D) or p21 (E–H), cyclin E (EL, T1, or T2), and CDK2. The levels of CDK2 and cyclin E (EL, T1, or T2) viruses were kept the same at a 1:1 ratio in titers in all tri-infected samples, whereas the levels of p27 (or p21) viruses increased, compared with titers of CDK2 virus, from 0, 0.25, 0.5, 1, 2, and 4 titers. Protein extracts obtained 60 h postinfection were subjected to Western blot (A and E), histone H1 and GST-Rb kinase assays (B and F), and immunoprecipitation analysis (C and G). For Western blot analysis, equal amounts (50 μ g) of proteins from each sample were loaded to SDS-polyacrylamide gels, and the blots were probed with the indicated monoclonal antibodies. For kinase analysis, 300 μ g of each protein extract were immunoprecipitated with polyclonal antibody CDK2, p27, or p21 and then coupled to protein A beads. Histone H1 or GST-Rb were used as substrates. Kinase bands were excised, and radioactivity was ascertained by a scintillation counter. Values were normalized against basal (no CKIs) activity and presented as graphs from which IC_{50} values could be derived (D and H). For immunoprecipitation analysis, 300 μ g of each protein extract were immunoprecipitated with polyclonal antibody CDK2, p27, or p21 and then coupled to protein A beads and subjected to Western blot analysis with the indicated antibodies. Data are representative of four replicate experiments.



cyclin E-CDK2 against GST-Rb was 25 nM (Fig. 2D). In contrast, the concentrations of p21 and p27 required to inhibit the kinase activity of the LMW cyclin E-CDK2 by 50% were 3–5-fold higher. The trend was similar when HH1 was used as a substrate (EL required less of each CKI to inhibit its activity by 50% compared with T1 and T2), but as would be expected, the differences between the isoforms was not as stratified due to the decreased specificity of the substrate. Furthermore, whether the raw CDK2-associated GST-Rb kinase activity (Fig. 2B) was used to determine the IC_{50} values or the normalized kinase results (Fig. 2C), the IC_{50} values were identical, suggesting that the resistance of the LMW forms of cyclin E to CKIs is not due to higher basal activity of these forms as compared with the full-length. Western blot analysis suggests that there was no significant difference in the binding of the p27 to the full-length *versus* the LMW cyclin E-CDK2 complexes (*i.e.* Fig. 2A, lanes 4–6). These results suggest that the LMW cyclin E is much more resistant to inhibition by p27 than the full-length cyclin E. Furthermore, the differences in sensitivity to inhibition by CKIs between the full-length and LMW

cyclin E is not merely due to the differences in the basal levels or activity of the LMW isoforms of cyclin E; nor is it due to differential binding properties of these proteins.

We next examined whether the resistance of the LMW forms of cyclin E to CKIs is evident in intact insect cells (Fig. 3). For these *in vivo* experiments, insect cells were tri-infected with cyclin E (EL, T1, or T2), CDK2, and increasing concentrations of p27 (Fig. 3, A–D) or p21 (Fig. 3, E–H). Sixty hours postinfection, cells were homogenized, and lysates were subjected to Western blot analysis (Fig. 3, A and E) with the indicated antibodies. Cyclin E-containing complexes were immunoprecipitated with p21, p27, or CDK2 and subjected to kinase assays using GST-Rb or histone H1 as substrates (Fig. 3, B and F) or Western blot analysis with p27 and CDK2 (Fig. 3, C and G). When cyclin E-EL, T1, and T2 are equally expressed and in the presence of equal levels of CDK2 (Fig. 3, A and E), the full-length cyclin E is exquisitely sensitive to p27 and p21, with a very low level of these CKIs leading to rapid and complete inhibition of the full-length cyclin E-CDK2 complex kinase function (Fig. 3, B and F). On the other hand, the LMW cyclin

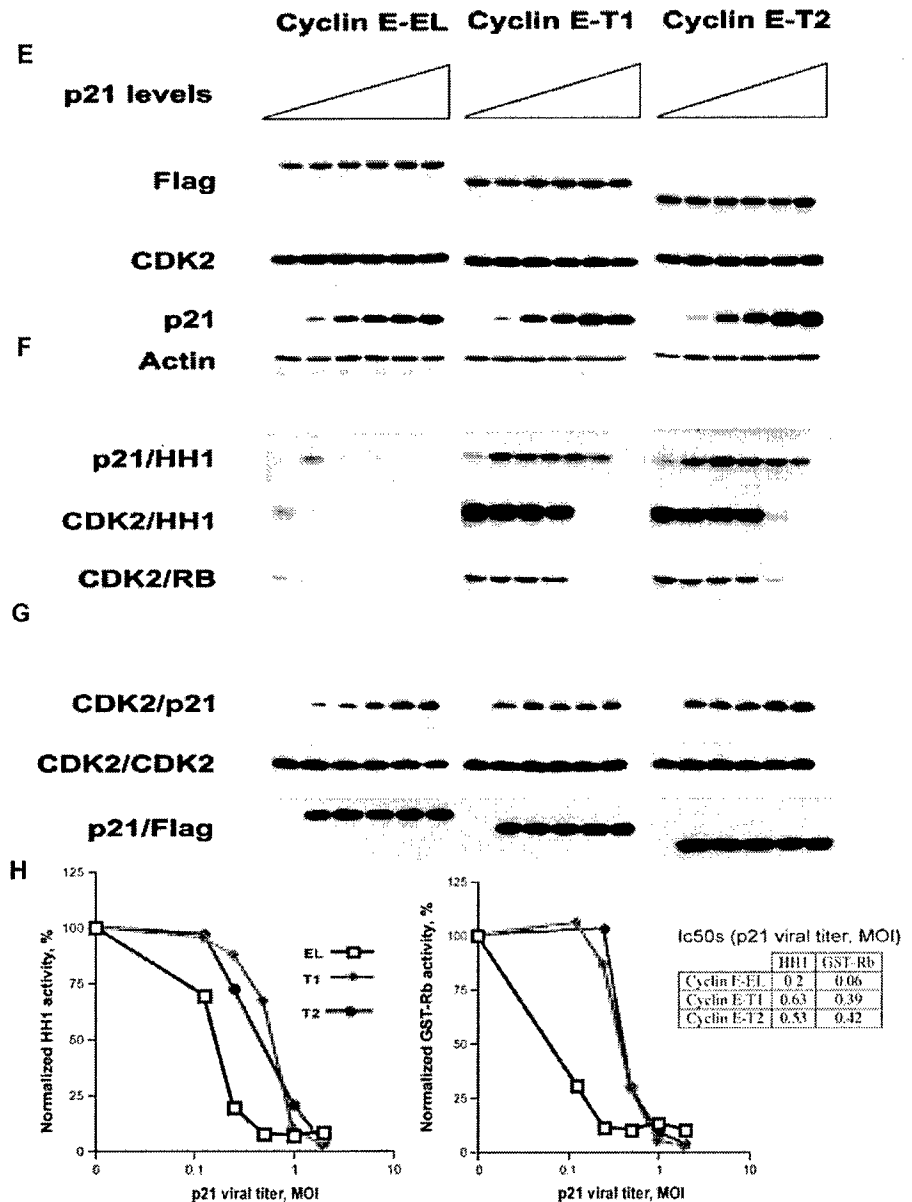


FIG. 3—continued

E-CDK2 complexes were significantly more resistant to inhibition by p27 or p21, requiring 8 times more p27 or p21 virus to effectively inhibit the LMW cyclin E-CDK2 kinase activity as compared with the full-length cyclin E (Fig. 3, *B* and *F*). Quantitative data were collected by scintillation counting the HH1 or GST-Rb bands from the kinase assay, and the activity associated with each isoform of cyclin E was normalized against the base-line activity, without p21 or p27, to account for any differences in their basal activity. The graphs of the normalized activity against GST-Rb and HH1 are shown in Fig. 3, *D* and *H*, along with the respective IC₅₀ values. The results revealed a striking resistance to p27 of the LMW isoforms of cyclin E associated activity toward phosphorylation of GST-Rb. The full-length cyclin E was inhibited to 50% with only an MOI of 0.18 of p27 virus, whereas 13-fold more (*i.e.* MOI of 2.3) was required for 50% inhibition of the LMW cyclin E-CDK2 kinase activity toward GST-Rb. Similarly, for p21 to inhibit the activity of the complexes associated with the LMW forms of cyclin E, 7-fold more virus was required than for the full-length cyclin E. These results clearly show that whereas the p21- and p27-

associated kinase activities were readily inhibited in cyclin E EL-containing complexes, their activity remained unaffected in the LMW form-containing complexes. In both the CDK2 and p27 kinase assays, the activity associated with the LMW forms of cyclin E is diminished only at the highest MOI of p21 or p27 virus. As we saw in the *in vitro* experiments, the binding of p27 or p21 to the cyclin E LMW forms in complex with CDK2 is not compromised, since equal (if not greater) amounts of p27 (Fig. 3C) or p21 (Fig. 3G) bind to complexes with the LMW forms of cyclin E compared with the full-length form (under conditions where equal levels of CDK2 were immunoprecipitated for each condition). Collectively, our *in vitro* and *in vivo* data suggest that, compared with the full-length cyclin E, the LMW forms of cyclin E are resistant to p21- and p27-mediated inhibition, contributing to the functional hyperactivity of these isoforms.

LMW Forms of Cyclin E Can Sequester p21 and p27 Away from the Full-length Cyclin E—In breast cancer cell lines as well as tumor tissue from breast cancer patients, the entire complement of the LMW forms of cyclin E is overexpressed along with the full-length cyclin E. In our experiments to date,

with the EL and the LMW cyclin E examined separately, we have seen that the LMW isoforms of cyclin E have higher affinity for CDK2 and are resistant to p21 and p27, resulting in their hyperactivity. We hypothesized that under conditions where both LMW forms of cyclin E and the full-length protein are expressed (as is the case in tumor cells and tissues; see below), the LMW forms can function to sequester the CKIs away from the EL cyclin E, resulting in a more effective G₁ to S phase transition. To directly examine this hypothesis quantitatively, insect cells were concomitantly infected with four different viruses: cyclin E-EL, cyclin E-T1 (or T2), CDK2, and p27. Sixty hours postinfection, cells were harvested, and cell lysates were subjected to Western blot analysis with the indicated antibodies (Fig. 4A). We also examined the kinase activity associated with each condition by immunoprecipitating with antibodies to CDK2, FLAG, or p27 (Fig. 4B). As expected, when insect cells were infected with cyclin E-EL, CDK2, and p27, p27 bound and inhibited the kinase activity associated with the cyclin EL-CDK2 complex (Fig. 4B, lanes 1–3). In contrast, insect cells infected with either cyclin E-T1 or cyclin E-T2 and CDK2, at two different titers of p27, were largely resistant to inhibition by p27 (Fig. 4B, lanes 4–6 and 10–12). Immunoprecipitation with p27, followed by probing with antibodies to FLAG, revealed that when both the LMW form of cyclin E and the full-length cyclin E (*i.e.* either cyclin E-EL + T1 or cyclin E-EL + T2) were co-infected into insect cells along with CDK2 and p27, p27 preferably bound to the LMW forms (lanes 7–9 and 13–15, compared with lanes 4–6 and 10–12; Fig. 4B). Therefore, despite preferential binding to the LMW forms of cyclin E, p27 does not inhibit the overall kinase activity associated with the LMW cyclin E-CDK2 complexes. The *asterisks* in Fig. 4C indicate the lanes in which densitometry was performed in order to quantify differential binding of p27 to the LMW forms of cyclin E compared with the full-length cyclin E. The ratio of cyclin E bound to p27 was determined for T1 *versus* EL, and T2 *versus* EL (Fig. 4D) through densitometric analysis of Fig. 4C, lanes 8 and 9 (EL *versus* T1), and Fig. 4C, lanes 14 and 15 (EL *versus* T2). This value was also normalized to the ratio of expression of the LMW forms compared with the full-length form in this system (Fig. 4A, lanes 7–9 for EL/T1 and lanes 13–15 for EL/T2). From this quantitative analysis, we found that T1 (lanes 8 and 9) and T2 (lanes 14 and 15) bind p27 on average 2.0 and 2.7 times more efficiently than does EL under conditions when both EL and T1 (or T2) are expressed simultaneously and at equal levels. These results suggest that by binding preferably to the LMW forms of cyclin E, p27 is being sequestered by these more active, yet CKI-resistant, forms of cyclin E. Therefore, the presence of the LMW cyclin E forms ensures that the total cyclin E kinase activity of the cell remains unaffected even under circumstances of high p21 and p27 expression.

The LMW Forms Are Hyperactive in Human Breast Cancer Cells Despite Efficient Binding to p27—Our results thus far suggest that the LMW forms of cyclin E may act as dominant partners to p27 compared with the full-length cyclin E. By binding p27 more efficiently than the full-length form, the LMW forms essentially sequester p27 from the full-length cyclin E. Furthermore, the LMW forms, despite binding significantly more p27 than the full-length form, are resistant to its inhibition. We suggest that similarly in tumor cells with deregulated cyclin E, the LMW forms of cyclin E act to sequester p27, maintain the activity of the cyclin E-CDK2 kinase complex, contribute toward abrogation of the G₁/S checkpoint, and thereby provide tumor cells with a growth advantage. We set out to test this hypothesis using two different systems: 1) in breast cancer cell lines transfected with each cyclin E isoform

(Fig. 5) and 2) in tumors from breast cancer patients overexpressing the LMW forms of cyclin E (Fig. 6). In the first system, we assessed whether the resistance of the LMW forms of cyclin E to p27 can also be seen in the human breast cancer cell line T47D. The mammary ductal carcinoma cell line T47D endogenously expresses low to undetectable levels of the LMW forms. We transiently transfected these cells with each of the isoforms of cyclin E (EL, T1, or T2), resulting in their strong overexpression compared with the endogenous cyclin E in untransfected cells or those transfected with an empty vector (Fig. 5). There were no significant changes in expression levels of the cyclin E-associated proteins CDK2, p21, and p27 in the cyclin E-overexpressing cells (Fig. 5A). However, the LMW forms of cyclin E in complex with CDK2 resulted in a striking increase in kinase activity compared with EL or the empty vector transfected cells (Fig. 5B). Furthermore, the LMW forms of cyclin E were complexed to at least equivalent levels of p27 as the full-length cyclin E (Fig. 5C). These results suggest that the LMW forms are efficiently binding to p27, but this is not sufficient for inhibition of their activity, further supporting our hypothesis that the LMW forms of cyclin E are hyperactive due to a differential sensitivity to CKIs compared with the full-length cyclin E.

The LMW Forms Are Hyperactive and Resistant to p27 in Human Breast Cancer Tumor Tissue Samples—To address whether the LMW forms of cyclin E are resistant to p27 *in vivo*, we examined the binding of p27 to the LMW forms of cyclin E in tumors from breast cancer patients. Fig. 6A shows a representative Western blot of nine tumor tissue lysates, five with low expression of the LMW forms of cyclin E and four with high expression of the LMW isoforms compared with the normal (76N) and tumor (MDA-MB-436) cell line controls. Those tumor lysates that had increased expression of full-length and LMW cyclin E also showed an increased expression of p27. Additionally, proliferating cell nuclear antigen levels were increased in those samples, with increased expression of cyclin E correlating with a higher proliferative rate. We observed that the p27 binds efficiently to all forms of cyclin E (Fig. 6B), resembling what we detected in insect cells (Figs. 3 and 4). We next determined the activity associated with the cyclin E-CDK2 complex of the tumor lysates (Fig. 6C). The tumor lysates that had high expression of the LMW forms showed increased kinase activity. Therefore, despite the presence of ample p27 and its efficient binding to the LMW forms of cyclin E, its resistance to p27 ensures an active cyclin E-CDK2 complex in tumor tissue samples from breast cancer patients.

DISCUSSION

In this paper, we have examined the biochemical properties of the low molecular weight, tumor-specific isoforms of cyclin E that lead to their hyperactivity as compared with the wild-type full-length form of the protein. Compared with the full-length cyclin E, the LMW isoforms bind more effectively to CDK2. Furthermore, the function of the full-length cyclin E-CDK2 complexes, using either histone H1 or GST-Rb as substrates, is readily inhibited by CKIs, p21 and p27. However, the LMW cyclin E-CDK2 complexes were significantly more resistant to inhibition by the CKIs, both *in vitro*, using purified p21 and p27, and *in vivo*, when the CKIs were co-infected with cyclin E and CDK2 vectors. This resistance is irrespective of the higher basal kinase activity observed in association with the LMW cyclin E complexes. Furthermore, this resistance to inhibition is not due to a compromise in the binding of p21 and p27 to the cyclin E LMW-CDK2 complex, since at least equal amounts of the CKIs bind to complexes with the LMW forms of cyclin E compared with the full-length form. Last, we show that when both the full-length and low molecular weight forms of cyclin E

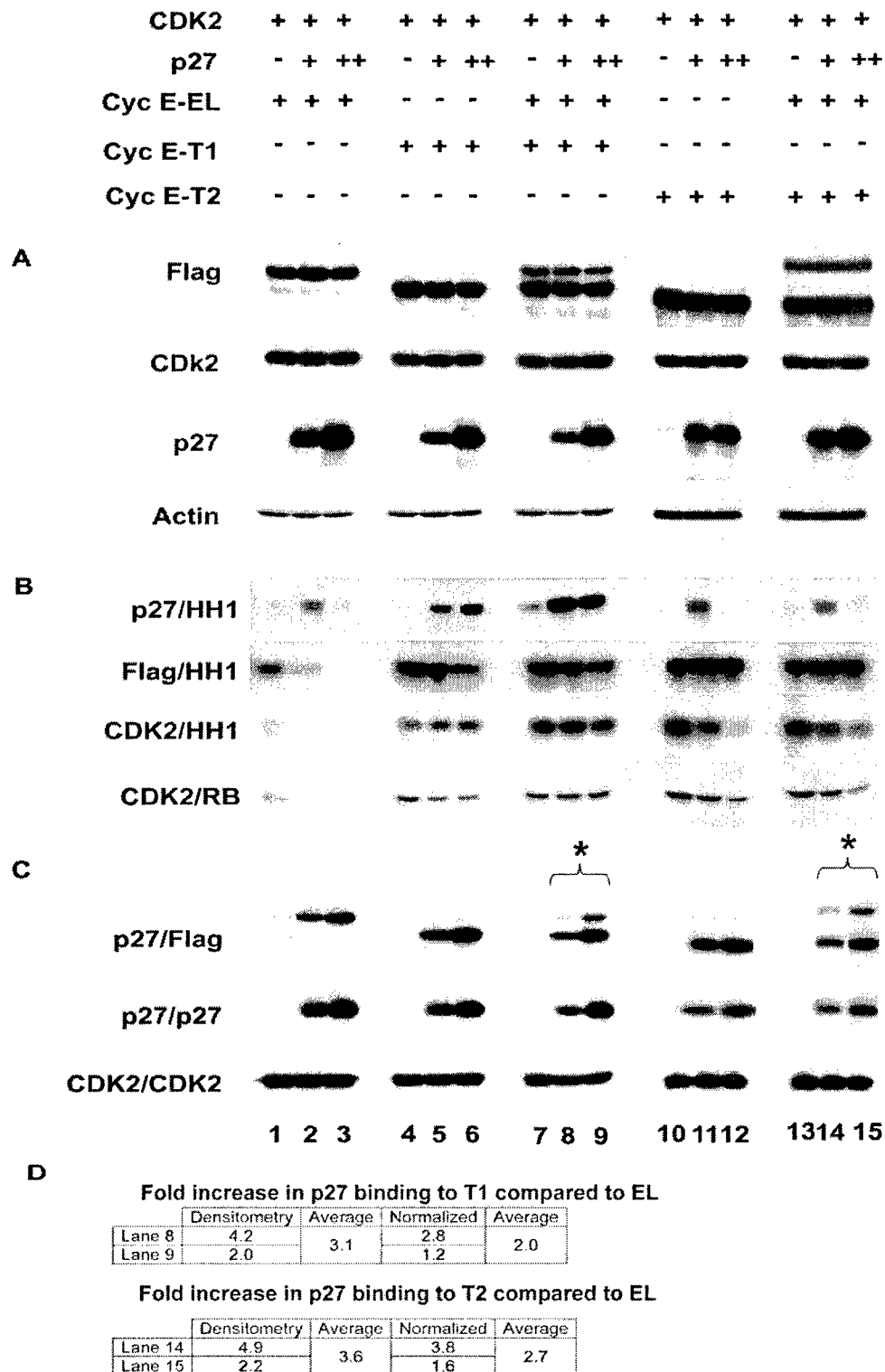


FIG. 4. The LMW forms of cyclin E (T1 and T2) can sequester the inhibitor p27 away from full-length cyclin E. Insect cells were simultaneously infected with one or more of the following BaculoGold viruses: CDK2, p27, cyclin E-EL, cyclin E-T1, or cyclin E-T2. Titers of CDK2, cyclin E-EL, cyclin E-T1, and cyclin E-T2 viruses were kept the same under all conditions. Titers of p27 virus increased in three MOI increments (0, 1, and 2 MOI of the titer of CDK2 virus) with each set. Protein extracts obtained 60 h postinfection were subjected to Western blot with the indicated antibodies (A), histone H1 or GST-Rb kinase assays, following IP with p27, FLAG, or CDK2 (B), and immunoprecipitation with p27 or CDK2 followed by immunoblotting with the indicated antibodies (C). The asterisks indicate lanes in which expression was quantified using densitometry, presented in D. The ratio of cyclin E bound to p27 was determined for T1 versus EL (C, lanes 8 and 9) and T2 versus EL (C, lanes 14 and 15). To establish a normalized value, this ratio was then divided by the ratio of expression of the LMW forms compared with the full-length form in this system (A, lanes 7–9 for EL/T1 and lanes 13–15 for EL/T2). This figure is representative of triplicate experiments performed.

are co-expressed in cells, p27 preferentially binds to the LMW forms and is unable to inhibit the CDK2 activity. Collectively, our data show that the LMW forms of cyclin E provide a growth

advantage in tumor cells by working as dominant forms of cyclin E, are resistant to inhibition mediated by CKIs, and sequester the CKIs away from the full-length cyclin E.

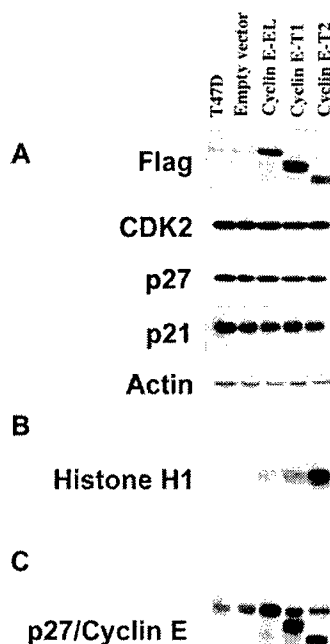


FIG. 5. The LMW forms of cyclin E bind to p27 and are hyperactive in T47D breast cancer cells. Three different cyclin E FLAG-tagged constructs representing the full-length (EL) and two of the LMW forms (T1 and T2) were transfected into the T47D breast cancer cell line, harvested 16 h post-transfection, and subjected to Western blot analysis (A), kinase analysis (B), and immune complex formation with p27 (C) as described under "Experimental Procedures."

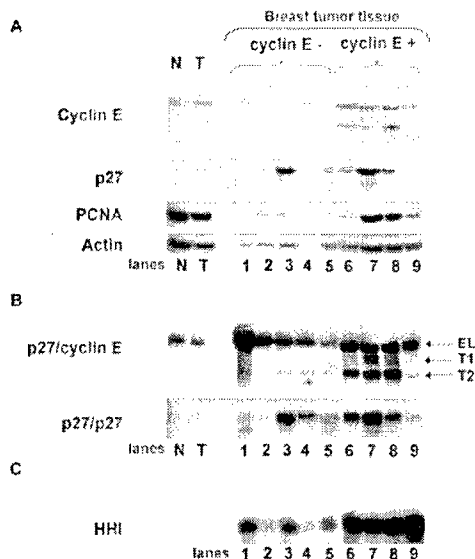


FIG. 6. p27 binds the LMW forms of cyclin E but does not inhibit their kinase activity in breast cancer patient samples. Tissue samples were collected from breast cancer patients with normal levels of cyclin E and cyclin E over-expressing tumors and then homogenized to lysates as described under "Experimental Procedures." 25 μ g of lysate were used for Western blots that were hybridized with the indicated antibodies (A). The control lanes correspond to cultured normal and tumor cell lines, where N represents the 76N normal cell strain and T represents the MDA-MB-436 tumor cell line. Immunoprecipitations were performed on 150 μ g of lysate using polyclonal p27 antibody coupled to protein A beads followed by Western blotting with the indicated antibodies (B). A kinase assay was also performed following immunoprecipitation with polyclonal cyclin E antibody and protein A beads using 150 μ g of protein extract and HHI as the substrate (C).

The observation that the LMW forms of cyclin E are more active than the full-length has significant implications for cancer cells. In normal proliferating cells, the levels of cyclin E are

tightly regulated such that it is expressed at the end of G₁ phase, and the levels decrease shortly after S phase (13, 14, 30, 31). In tumor cells, on the other hand, cyclin E is deregulated in several ways. First, the levels of the full-length cyclin E are often overexpressed and present constitutively in the cell cycle, resulting in unabated phosphorylation of key substrates such as pRb (16–19), which gives the tumor cells a growth advantage as the "go signal" mediated by cyclin E. Second, the full-length cyclin E is cleaved by the elastase class of proteases to generate the LMW forms of the protein (23, 28). Here we show that these LMW forms of cyclin E not only are more active than the full-length cyclin E, but they are more resistant to inhibition by both p21 and p27. An interesting observation is that at the highest concentrations of inhibitor used, there is an abrupt inhibition of the LMW forms (Fig. 4, A and B), suggesting that there is a threshold effect in place. Therefore, it appears that in cells that express LMW cyclin E, significant overexpression of p27 is required to effectively inhibit the total cyclin E-CDK2 kinase activity of the cell.

The difference between the full-length and the LMW forms of cyclin E is the deletion of 40–70 amino acids at the amino terminus of the LMW isoforms (23). We have previously shown that the LMW isoforms are generated by proteolytic cleavage at amino acids 40–45 (EL2 and EL3) and at amino acid Ala-69 (EL5 and EL6) (23). The sequences surrounding these cleavage sites are both short hydrophobic stretches of amino acids followed by aspartate-proline, and both are embedded in a random coil secondary structure (32). Therefore, the structure is of two hairpin loops at the N terminus, which is similar to that of the serine protease inhibitors plasminostrepsin and *Streptomyces* subtilisin inhibitor, making these sequences accessible to the protease (33). Here we have shown that p27 can bind the LMW cyclin E-CDK2 complex. However, we predict that there is a physiological threshold of cyclin/CDK2 activity for which p27 can inhibit and that the hyperactivity resulting from the increased affinity of the LMW cyclin E for CDK2 is above a threshold of which p27 can inhibit. Work is currently in progress to delineate how the amino-terminal sequences of cyclin E can bind and facilitate inhibition by CKIs.

The role of p27 protein as a negative regulator of the cell cycle and positive prognostic indicator is well established. Reduced expression of the p27 protein has been reported in several human tumors and has been associated with increased mortality and poor prognosis (34–38). However, the increased expression of p27 protein has also been observed in many colon and breast cancer tissues (37–41). The question raised by these studies is how p27 can be inactivated such that it can no longer act as a negative regulator of cell proliferation in these invasive cancers. Two different mechanisms leading to p27 inactivation have previously been reported. Several studies have shown that the sequestration of p27 into the cytoplasm is one way to inactivate the p27-associated inhibitory activity (40, 43). Additionally, cyclin D1 has been reported to act as a sink for p27 by sequestering p27 away from cyclin E-CDK2 complexes to cyclin D1-CDK4 complexes (44–48). Our results provide another means of p27 inactivity. Our data suggest that the LMW forms of cyclin E can also act as a sink for p27. Hence, tumor cells gain an additional growth advantage; under conditions where high levels of p27 are expressed, the LMW forms of cyclin E can bind to p27 and abrogate its role as a negative regulator. Such sequestration of p27 by the LMW forms of cyclin E occurs in the nucleus, since the LMW cyclin E are only localized to the nucleus (23). Therefore, the absolute expression levels of p27 are probably less important prognostically than the complexes formed by p27, shedding some light on the conflicting reports concerning strictly the relative ratio of complex formed be-

tween p27, CDK2, full-length cyclin E, and LMW cyclin E. These complex interactions between p27 and other G₁/S regulators probably accounts for the conflicting reports concerning p27 levels and breast cancer (35, 37, 49, 51, 52).

Last, the LMW forms of cyclin E could provide a novel target for future drug design for the treatment of metastatic breast cancer, without harming normal proliferating cells in the body. Our studies have implicated a protease that is induced during metastatic progression (42, 50, 53) in the proteolytic regulation of cell cycle progression. By identifying the specific protease (*i.e.* of the elastase class) that cleaves cyclin E into its LMW forms found exclusively in tumor cells and tissues, we may be able to design cyclin E-specific protease inhibitors. These inhibitors could then help control the progression through the cell cycle of invasive cells, thus limiting the ability of these cells to populate distant metastatic sites.

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